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Technical Bulletin No. 58 December, 1922

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IMPERIAL BUREAU
21 MAR. 1923
OF MYCOLOGY.

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The Occurrence of Protozoa in Plants Affected With Mosaic and Related Diseases *

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GENERAL CONSIDERATIONS

The mosaic diseases of plants have occupied a prominent place in the field of phytopathological research for many years. Their increasing economic importance and wide distribution have attracted the attention of more and more workers in this country and abroad, and today they constitute a major project for investigation in most of the institutions where botanical research is carried on. For several cultivated crops the mosaic diseases of the infectious type are the most serious diseases known. Much progress has been made along the lines of inter-relationships, modes of transmission, overwintering, etc., but, despite the large amount of work done, the etiology of not one of the group has advanced much beyond the realm of speculation. The enzyme theory has its followers, and there are those who lean toward a belief in the bacterial nature of the causal agents, but apparently the concensus of opinion among plant pathologists is, that the mosaic diseases are analogous to certain animal disorders caused by the so-called filterable viruses. Probably no single factor has so retarded research into the nature of the etiological factor of these diseases as the general belief that the organisms are so small that they can pass through filters, the diameter of whose pores is smaller than the visual magnifying powers of the microscope. While the trend of evidence has seemed to indicate that the particles of infectious material in the form that they are extracted from the tissues of diseased plants are very small, there is little justification for the conclusion that these filterable forms represent the only ones capable of producing the disease. The extreme polymorphism that is characteristic of the life-cycles of many of the protozoa and our lack of knowledge concerning filtration through pressure and suction filters, open for the investigator in mosaic diseases fields for research which have as yet been unexplored.

Recently interest has been aroused through the work of Matz (1)†, Kunkel (2), and Palm (3), who respectively, have found foreign bodies in the cells of sugar cane, corn, and tobacco plants affected with mosaic. Although no definite organisms have been demonstrated in the tissues of any plant affected with mosaic or the closely related diseases, the work in progress indicates a general belief by many workers in the occurrence of such a factor.

*The writer is greatly indebted to Dr. G. H. Coons for very valuable suggestions given during the course of this investigation. He also acknowledges the helpful criticism of Dr. E. A. Bessey and his aid in the interpretation of the slides.

†The number in parenthesis refers to the number of the article in the bibliography on page—of this bulletin.

Symptoms of Diseases of This Type

Many of the economically more important plant families contain individuals or groups of plants known to be affected with mosaic disease. More than twenty families have been so recorded. The symptomatology is strikingly similar for nearly all these plants, suggesting a close relationship among the causal agents. With but slight modification or amplification the descrip-

FIG. 1. Bean plant affected with mosaic, showing the typical mottling and distortion of foliage. Slightly reduced.

tion of mosaic symptoms on any plant can be made applicable to any other plant of a different group. (See Fig. 1, Bean Mosaic.) In general, typical symptoms include: mottling of the foliage in darker and lighter areas, crinkling of the leaves, or other distortional modifications, dwarfing, reduction in

yield, and communicability of the etiological factor by contact or through the intervention of insects, mainly the Aphididae. Wilting, chlorosis, leaf drop, canker formation, are symptoms specific for certain mosaic diseases but are not generally applicable to the group.

Histological Lesions in Related Diseases

Quanjer (4) has found and described a definite necrosis of the phloem tissues in potato plants affected with leaf-roll. His findings have been confirmed by Artschwager (5). No investigation of the phloem tissues of mosaic plants has so far been reported, and apparently the significance of the association of necrotic tissues in a disease so closely related to mosaic as leaf roll has not been appreciated. In disease phenomena, cause and effect are usually closely associated, so that it seems reasonable to assume that the finding of certain areas specifically affected by disease indicates also the location of the inciting cause. The location in the phloem of the agents which cause the mosaic diseases is further indicated by observations on mode of transmission.

Modes Of Transmission

The mosaic diseases, and others, like potato leaf-roll, are transmissible by insects. Plant lice are mainly responsible for the widespread dissemination of the infectious material. The feeding habits of these insects offer valuable evidence as to the probable nature and location of the causal organisms. Those species known to transmit these diseases take their food material from the phloem tissues of the host plants, and after feeding for some time upon the veins and parts of the plants close to the phloem they are capable of transmitting the disease to healthy plants. Other species which do not attack the food-conducting tissues have not been shown consistently to be carriers of the infectious agents.

The fact that some of the mosaic diseases are contagious indicates that the causative agents are generally distributed in the plant. Juice expressed from any aerial part of the plant is usually effective in reproducing the disease when inoculated under favorable conditions into healthy plants. A cytological study employing modern improved methods, in a search for possible causative organisms in the phloem of diseased plants has not been reported. Investigators have reported meagerly upon observations of tissues in mosaic plants but no exhaustive search has been made of the phloem, apparently the vulnerable point of attack in seeking for the solution of the true nature of the mosaic organisms.

PRESENT INVESTIGATIONS

Bean mosaic investigations have been in progress at the Michigan Agricultural Experiment Station since 1919. Most of the work has been concerned with variety and species susceptibility, and with insect transmission. Some cytological work was done during 1921 but was not pursued to any conclusion. As soon as mosaic material was available in the summer of 1922 an intensive histological study was projected, using all of the common killing and fixing solutions and botanical stains. The main object of the investi-

gation was to determine whether or not any organisms could be demonstrated in the sieve tissues of mosaic plants. As the bean work progressed the scope of the investigations was widened to include tomato mosaic, clover mosaic and potato leaf-roll.

Materials

Bean mosaic material has been available in great quantities from a mosaic seed plot which has been grown each year as part of the field experimental work. This has included most of the common varieties of field and garden beans, as well as Lima, yard long, adsuki, rice, urd, tepary, moth and Windsor beans. All of these species showed distinct mosaic symptoms during the summer of 1922, which was a very favorable season for the development and spread of the disease. For a study of healthy tissues, seed from healthy plants was grown under cheese cloth cages in the greenhouse.

Tomato mosaic was common late in the summer and material was collected from several gardens where practically all of the plants were diseased. Potato leaf-roll was not found in great quantity but sufficient typical material was secured for the purpose of this investigation. Clover mosaic was plentiful and was used as a check upon the results obtained in studying bean mosaic, since it has been shown that the mosaic disease of each can be reciprocally transferred.

Methods

In all of the early work on bean mosaic the ordinary botanical histological methods were followed. Paraffin sections were stained with the stains most used by botanists. Many thousand transverse sections were cut of leaf, stem and petiole material imbedded in paraffin and studied under the high powers of the microscope, but, aside from slight deviations, diseased plants revealed nothing more than healthy ones. It was then decided to try other killing and fixing agents and employ new stains. Various solutions used by protozoologists were modified to adapt them to plant material, and protozoan stains were used for coloring the sections. Small bits of stems, leaves and petioles were taken from near the growing points of the plant and fixed for 12-24 hours in the killing and fixing solutions. The material was then washed for 24 hours in running water, dehydrated and imbedded in paraffin in the usual way. A radical departure in technique, and one which it is believed would have illuminated the mosaic problem long ago if employed, was introduced at this point. Longitudinal sections 10 microns thick were cut from the paraffin material. From the photomicrographic illustrations in works published on mosaic and related diseases it appears that the basis of these studies has been mainly the study of transverse sections. Apparently little work has been done in which longitudinal sections have been stained and critically examined. There are probably various reasons for this. It is very difficult to cut longitudinal sections of stem and petiole material due to the lignification of the xylem elements and the presence of fibrous tissue in the phloem. Much patience and a perfect condition of the sectioning razor are essential to success in securing sections that are not badly mutilated. Since the general assumption has been that the organisms were very small and generally distributed throughout diseased plants, the conclusion that transverse sections should contain them was sound from this standpoint. However, the results of the work presented here show why the cross section method has failed. Transverse sections have proved, in the author's work, to be without value so far as showing the location, position or nature of the organisms.

Longitudinal sections are apparently indispensable and have revealed structures not demonstrable by any other method.

Some erroneous views held by botanists have been dispelled by observations of many thousands of sections of bean, tomato, potato, and other plants. Some botanists have taught that nuclei are not present in the sieve tubes. Nevertheless, they occur regularly in sieve tubes in all stages of development and are easily demonstrated by a study of longitudinal sections. Plastids, which are also supposed to be absent from the sieve tubes, are often found in great numbers. This is especially true of cucurbitaceous material. In mosaic plants the plastids may almost surround the nucleus in cells of the sieve parenchyma and adjoining tissues. Abnormal nuclei are also present throughout the phloem of diseased plants and may easily be mistaken for possible organisms by any one not familiar with the material. Chondriosomes are numerous in the parenchyma cells of some of the plants studied and due to their great resemblance to large bacteria, especially in stages of division, are susceptible to erroneous interpretation.

ORGANISMS IN DISEASED PLANTS

Bean Mosaic

The sieve tubes of bean plants are remarkably free from food particles that take the stains used in this work. This is not true with all plants since sections of mosaic material from plants of other kinds have shown these tubes to be filled with deeply staining granules of appreciable size. Because of the clarity of the phloem tissues when properly destained the bean offers excellent material for a study of this kind.

Longitudinal sections of stems or petioles from mosaic plants, killed and fixed in protozoan killing and fixing solutions[1] and stained with Haidenhain's iron-alum haematoxylin, show the constant occurrence of biflagellate organisms in the sieve and sieve parenchyma cells. They are fixed and stained powerfully by these solutions and by proper destaining all color can be removed from all plant structures except the nucleoli and chromatin and still remain brilliant in the organisms. The organisms are relatively large, some individuals being 18 microns in length, and are easily seen with ordinary high power dry lenses. Including the fully extended flagella some individuals will measure 50 microns. These flagellates are scattered throughout the sieve and adjoining parenchyma cells in the stems and petioles of diseased plants. They could not be found in the tissues of healthy plants, of which many were studied, using the same methods of killing, fixing, dehydrating, sectioning and staining. Fig. 2 is a photomicrograph of a longitudinal section through petiole phloem of a diseased plant and illustrates the distribution of the organisms.

The following are the principal forms that have been observed in sections of diseased tissues: (1) Large, elongated, biflagellate, tapering, or cigar-shaped individuals with the form of the body resembling organisms belonging to the genus Leptomonas, the flagella being attached at opposite poles. This is the most common type. (2) Ovaliform biflagellates of the same type. (3) Deep-staining, slightly elongated bodies, surrounded by a lighter en-

[1] Various standard solutions containing $HgCl_2$, were used.

velope, with flagella running down to the centrally stained portion and projecting from each end of this destained envelope. Individuals of this type may be almost spherical and occur in pairs in the same cell. (4) Very small, elongated flagellates, less than 0.5 micron in diameter and similar to type 1. These are common in sieve and parenchyma cells and probably represent very young forms of type 1. (5) Deeply stained oval bodies with degenerate flagella and occasional remnants of an outer envelope. These may be encysted forms. (6) Small, elongated, non-flagellate bodies which occur in great numbers in the degenerate chloroplasts of the sub-epidermal cells of

FIG. 2. Longitudinal section through the phloem of bean plant affected with mosaic, showing flagellated organisms lying in the sieve tubes and sieve parenchyma cells. × 650.
A. Body of the flagellates.
B. Flagellum.

diseased stems and petioles. They may entirely fill the cell lumen by their escape from the chloroplasts or remain imprisoned within them (Fig. 7). These organisms are very variable in size but are much smaller than the flagellate forms. Since a detailed study will have to be made of all these types before they can be assigned a place in the life history of this organism they are given only brief mention here, and will be described fully in a future publication. Such diversity of form indicates the probability of extreme

polymorphism in the life cycle. Some of these may be transitional forms and others may be merged upon further study.

Types 1 and 2 are shown in Fig. 3, and another individual of type 1 more highly magnified is seen in Fig. 4. The flagella may project in the axial line from the body of the organism or they may emerge slightly to one side of the axial line. The flagella extend into the body of the organism for a very short distance and seem to have their origin in blepharoplasts or basal granules which are located near the extreme ends of the flagellate. These basal

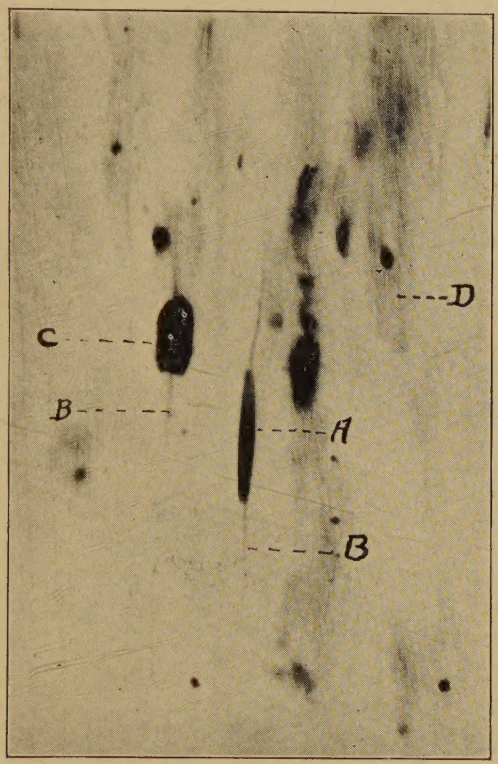

FIG. 3. Photomicrograph of biflagellate, elongated protozoan in sieve tube of mosaic bean plant × 1500.
A. Elongated organism of type 1.
B. Flagellum.
C. Ovaliform flagellate, type 2.
D. Cell nucleus with deeply-stained nucleolus in center.

granules take the stain more intensely than the rest of the organism and are easily located.

The nucleus is apparently little affected by the haematoxylin stain, although an occasional one, faintly colored, is seen centrally located in the cell. Special stains will have to be used to bring out details of structure. So far the polychrome methylene-blue stains and their modifications have not given satisfactory results with plant material. The main difficulty seems to be that destaining or dehydration in alcohol removes the stain from the organisms as well as from plant structures.

The majority of these flagellates lie in a plane parallel to the long axis of

the host cell. Their length would in most cases prevent their assuming any other position, although smaller forms are sometimes found in an oblique position. The flagella are extended in the majority of cases but may be coiled in various ways. The full extension of the flagella seems to be correlated mostly with the position of the organisms in relation to the host cell nucleus. In nearly every case the flagellates will be found to lie very close to the nucleus with one or both flagella in contact with the nuclear surface, or else the body of the organism is located parallel to the axial line of the

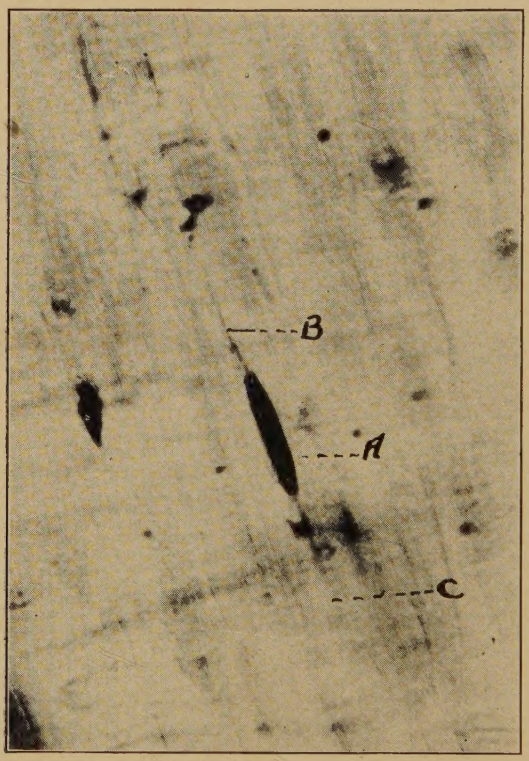

FIG. 4. Photomicrograph of a single biflagellate organism in a sieve parenchyma cell of bean petiole. × 1800.
A. Body of the flagellate.
B. Flagellum.
C. Sieve parenchyma cell.

nucleus and one flagellum is coiled around it. In one very interesting case the entire body of the flagellate lay in contact with the nucleus, the flagella being fully extended from both poles. Nuclei in various stages of degeneration occur throughout the phloem tissues and there seems little doubt of the direct influence of the presence of the flagellates upon the activities of this structure.

The mode of division of the biflagellate forms is by simple, longitudinal binary fission. Preceding the division of the organism the basal granules divide, the flagella split throughout their entire length or divide only partially, a v-shape slit appears in each end of the body and division then oc-

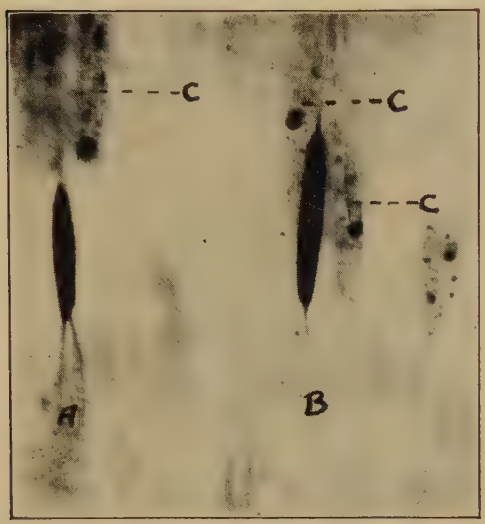

FIG. 5. Photomicrograph of longitudinal section through leaf petiole of mosaic bean plant, showing first stage of division of flagellate and proximity to host cell nucleus. × 1500.
A. Division of flagella preceding longitudinal binary fission into two flagellates.
B. Biflagellate organism lying in contact with host cell nucleus.
C. Nuclei with dark-staining nucleoli in center.

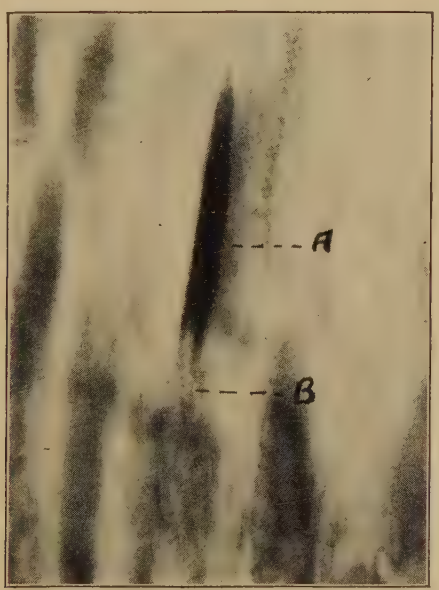

FIG. 6. Flagellate from bean plant dividing by longitudinal binary fission. × 1800.
A. Two daughter flagellates.
B. Flagellum partially divided.

curs, separating the organism into two equal daughter flagellates. These stages of division are shown in Figures 5 and 6. It is very likely that other modes of division occur in the life history of this organism. No form of division which produces the very small flagellates, some less than 0.3 micron in width, has been observed. Whether the large, elongated forms represent the adult type, which would be the basic form for generic classification, or whether some of the other types are the real adults, are questions which can not yet be answered. The form in which the organism is inoculated into healthy plants

FIG. 7. Photomicrograph of longitudinal section through subepidermal tissue of bean plant affected with mosaic. Note that the chloroplasts are filled with small, elongated, deeply-stained organisms. × 1500.
A. Chloroplasts filled with the organisms.

by the aphids which transmit the disease, and the question of whether or not these insects are merely accidental carriers of infectious material or actual hosts for some form in the life cycle of the flagellates are questions whose answer is merely speculative at present.

The migration of these flagellates from cell to cell has not been observed. The small forms might readily pass through the perforations in the sieve plates and thus become distributed throughout the sieve tubes. Likewise the very small flagellates once introduced into the parenchyma cells could possibly pass from cell to cell through the cytoplasmic connections. Further study is necessary for clarification of these points.

The organisms have been demonstrated in living material by means of thin longitudinal sections through the phloem and also by dark field illumi-

nation of juice expressed from diseased petioles. The first method is the most satisfactory. Petioles from plants showing severe mosaic symptoms were used and longitudinal sections cut through the phloem after splitting the petiole with a Gillette razor blade. These sections were mounted immediately in boiled water and examined under the high-power dry lenses, the illumination being reduced to a minimum necessary to make out plant structures. The flagellates were seen in the sieve tubes in an actively motile state. In stained as well as living material rarely is more than one individual found in a cell, and some cells contain none, so that it is necessary to find a favorable location on the slide where the flagellates are present. The body of the organism is seen in a rapidly whirling motion, disappearing from sight in the depths of the cells only to reappear again, turning over and over but changing its relative position but slightly in the cell. Only the body of the flagellate can be seen, as the flagella are not visible under these conditions.

For examining the organisms with dark-field condenser short pieces of petiole were laid on clean plate glass and the juice rolled out by means of a photographer's hard-rubber roller. Hanging drops were examined under oil-immersion lenses with arc-light illumination. An occasional flagellate was seen to flash across the field but apparently so few organisms are expressed in the relatively large amount of juice that they are not easily located in this way. Thin longitudinal sections are more satisfactory for observing the motility.

The size of the flagellates is very variable, as would be expected where they are present in various stages of development. Measurements of type 1, the elongated biflagellate forms, which constitute the majority of individuals, are given here. These measurements are based on fixed and stained material and, therefore, will be slightly less than the actual size of the organisms in a living state. A filar micrometer was used in making the measurements and the size of the body determined separately from the flagella. The following table shows the variation in 50 individuals measured in consecutive order as they occurred in the sieve cells:

Longest individual	18 × 2.8 microns.
Shortest individual	5 × 0.3 microns.
Broadest individual	13.9 × 3.9 microns.
Slenderest individual	11.3 × 0.3 microns.
Longest flagellum	18 microns.
Shortest flagellum	7.2 microns.
Average length of organism	13.5 microns.

The generic position of these flagellates does not coincide with that of any of the known protozoa. Before fixing their systematic position decision must first be made as to which form represents the adult type. Individuals with a flagellum attached to each end of the body are most common and appear to represent the adult form, and if generic classification is based on their morphology, then it will be necessary to erect a new genus to include them, as they differ from those genera of protozoa at present known which possess a single flagellum attached to each pole. Mention should be made here of the possible relationship but not generic identity of these organisms to those found in the latex of Euphorbiaceous and Asclepiadaceous plants and belonging to the genus Leptomonas (6). The bean organism is distinctly a different type, however, and its exact position will necessitate further study.

Clover Mosaic

In order to determine if organisms similar to those found in bean plants affected with mosaic were also present in clover plants showing mosaic symptoms, stems and petioles from affected plants were fixed and sectioned in the same way as the bean material. Unfortunately several heavy frosts had occurred before this material was collected and it therefore was not so satisfactory as would have been collections made earlier.

Examination of stained sections showed that the same type of flagellates that were present in diseased bean plants were also present in mosaic clover plants. Since it has been demonstrated that clover mosaic may be transmitted to the bean, producing typical bean mosaic, and vice versa, the finding of similar organisms in mosaic clover and mosaic bean strengthens the probability of a causative relation between the bean flagellate and the disease with which it is associated. The organisms in the clover plants were not

Fig. 8. Photomicrograph of longitudinal section through phloem tissue of clover plant affected with mosaic. A single elongated flagellate is seen lying in a sieve tube. × 1500.
A. Body of the flagellate.
B. Flagellum.
C. Sieve tube.

found in great numbers and the most of them had apparently been destroyed by the freezing temperatures to which the plants had been subjected. Disintegrating forms were quite numerous in the sieve tubes and non-flagellate individuals were found in greater numbers than flagellate forms. Fig. 8 shows, lying in a sieve tube, a single individual of the same form as type 1 of the bean flagellate. The small biflagellate forms and an occasional broad form found in the bean were also present in clover. The organisms were almost invariably located close to the nucleus and often in contact with it.

No extensive study was made of the clover mosaic material, but from the sections examined there seems little doubt of the identity of the flagellates

found in diseased plants with those associated with bean mosaic. No measurements were made but in size the clover organisms seem to conform with those measured in the bean.

Tomato Mosaic

No great difficulty was encountered in locating, in the sieve tubes of tomato plants affected with mosaic, organisms which, for this disease, are as constantly associated and as typical as the biflagellates found in bean and clover. In longitudinal sections of stems and petioles, fixed and stained with the solutions used in the study of bean mosaic material, these organisms are found scattered along in the sieve tubes as deep-staining, long, sinuous, tapering bodies, with one or both ends drawn out to a fine point, and usually with

FIG. 9. Longitudinal section through phloem tissue of mosaic tomato plant. × 650.
A. Two trypanosome-like organisms lying side by side in a sieve tube.
B. Two of the organisms attached to each other by posterior ends of the body. This is probably the phenomenon of agglomeration.
C. An organism apparently passing directly through walls of the sieve tube.
D. Sieve tube.

one end larger than the other. There is great variation from long and slender to short and relatively broad types, and individuals are present which apparently are in various stages of development. Their location in the sieve tubes is not constant but the majority of them lie in proximity to the host cell nucleus. In all cases, however, the organisms lie in a plane parallel to the longitudinal axis of the cell. For this reason cross sections of tomato are of as little value, so far as finding the organisms is concerned, as are similar sections from the bean, and if by chance a transverse section should sever

one of them, the nature of the organism would be difficult to determine. The organisms hold quite tenaciously to the haematoxylin stain, and if the sections are mordanted for 6-8 hours in the iron-alum solution, after prolonged destaining they still will be stained deeply while all color will be removed from plant structures except the nucleoli and chromatin. They are not so numerous nor do they occur in an unbroken succession of cells as was often found to be the case with the bean flagellates. They seem rather to occur in "nests," that is, at a certain place in the stem or petiole they will be quite numerous, then for a short distance they will not be found Several individuals may lie in one sieve tube, then for the distance of several cells none will be present. They have been found, however, in all parts of the stem and petiole from which sections have been cut.

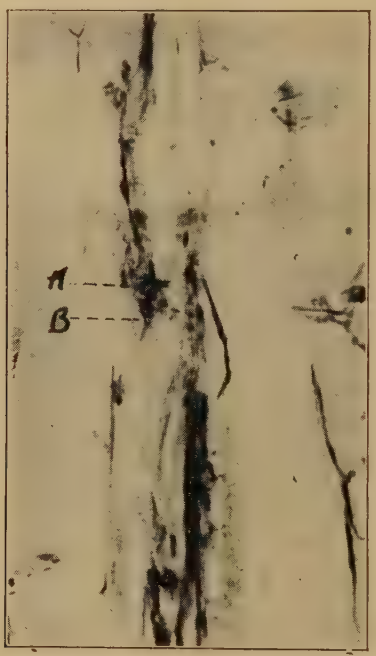

FIG. 10. Photomicrograph of a sieve tube in a mosaic tomato plant showing a small trypanosome passing through a perforation in the sieve plate. × 1500.
A. Sieve plate.
B. Trypanosome.

Fig. 11 shows the typical trypaniform nature of the organisms as they are found in the sieve tubes, while a broader type is seen in Fig. 12. An occasional colony of very small individuals has been seen, the most interesting case being where about eight apparently young individuals were found lying very close to a sieve plate. Division of some larger form or repeated division of the smaller type had apparently produced the increased number. The location of these very small individuals close to the sieve plates is correlated with migration from cell to cell. These small forms have been seen passing through the perforations in the sieve plates. In Fig. 10 is shown one of these small organisms half way through one of these holes in the sieve plate. The only type of division observed was by fission. Longitudinal binary fission

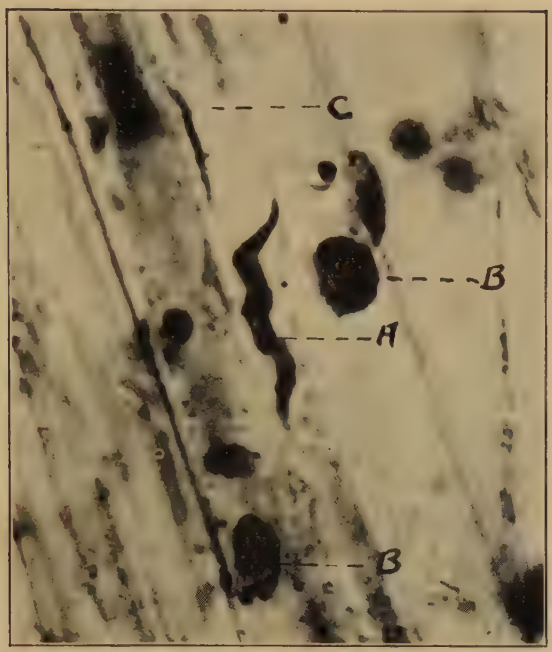

FIG. 11. Longitudinal section of phloem tissue in tomato plant affected with mosaic. A trypanosome is seen in a sieve tube. × 1500.
A. Trypanosome.
B. Cell nucleus.
C. Sieve tube.

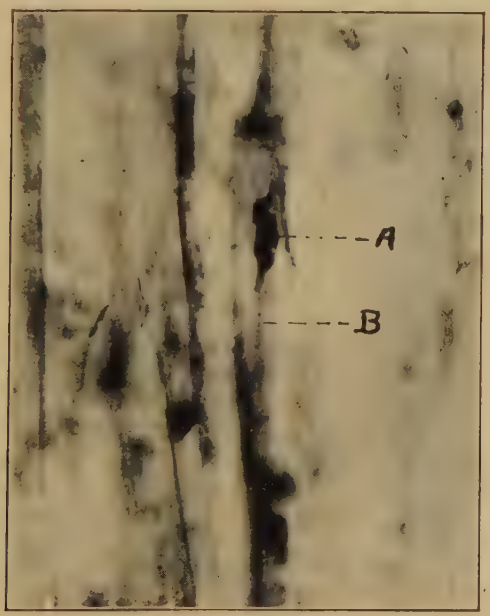

FIG. 12. Photomicrograph of a broad type of organism in a sieve type of a mosaic tomato plant. × 650.
A. Trypanosome-like organism.
B. Sieve tube.

was observed in several cases, but only of the small forms. Other types of multiplication probably occur, for undoubtedly the large forms split up into a number of smaller individuals.

The organisms are not invariably of the long sinuous type; many individuals show only a slight undulation of the body and others are almost straight. The slightly sinuous type of organism is shown in Fig. 9, which is a photomicrograph of a longitudinal section through sieve petiole tissue. In this same figure is illustrated what probably is the phenomenon known as agglomeration, that is, two or more organisms attached to each other by the posterior ends of the body. Among protozoa parasitic upon animals especially

Fig. 13. Photomicrograph of a very small protozoan closely applied to a nucleus, which is destained. × 1500.
A. Protozoan.
B. Destained nucelus.
C. Deeply stained nucleus in adjoining cell.

trypanosomes, agglomeration of individuals is a very common phenomenon. It is supposed to be analogous to agglutinization of bacteria under unfavorable conditions. At first the observation of these organisms in this position is likely to suggest conjugation, but since no definite evidence is available to show that this ever occurs among organisms of this type the rather frequent finding of them in this position is interpreted as representing the occurrence of agglomeration. No explanation is suggested as to its cause, but possibly fixation may be responsible.

The size of the organisms varies as widely as do young and old individuals of other protozoa. A very small form is shown in Fig. 13. This is apparently

a young organism and it is lying in contact with the host cell nucleus which is destained. Photographed at the same magnification a large individual is shown in Fig. 14. These two organisms illustrate the observed range in size. The figures given in the following table are indicative of the variation found among the organisms but are not to be interpreted as representing conclusive or final sizes of the organisms, for they are not based on measurements of a sufficiently large number of individuals:

Longest individual measured	28.2 microns.
Shortest individual measured	6.2 microns.
Largest individual measured	27 × 6 microns.
Smallest individual measured	6.2 × 0.5 microns.
Average all measurements	15.3 × 2.8 microns.

The total length of these organisms is very difficult to determine because of

FIG. 14. Very large trypanosome in a sieve tube of mosaic tomato petiole tissue. × 1500. Note comparative size with small form in Fig. 12, same magnification.

the sinuous form of the body. The above measurements were made from one extremity of the body to the other of each individual and no attempt was made to ascertain total length. The sizes are, however, comparative for all types.

The organs of locomotion have not been determined with certainty. An undulating membrane has been seen faintly stained in some of the forms studied, but no definite posterior or anterior flagellum has been demonstrated. The undulating membrane appears to run nearly the entire length of the body and has only been seen on some of the long, sinuous forms. There is such variation shown by the forms found in the sieve tubes—some

being very long and sinuous, others almost straight, and still others comparatively short and broad—that the morphology of the species will require extensive investigation. Such polymorphism, however, as was shown by the bean flagellates has not been found in these organisms associated with tomato mosaic.

In the slides stained with haematoxylin only a very few of the organisms have been found with the nucleus sufficiently stained to make it visible. In some of the preparations where destaining was carried far enough it can be made out centrally located in the body, while other deep-staining granules, apparently kinetonuclei, are visible near the extremities. Some portions of the organisms are stained more deeply than others but for a detailed study of structure it will be necessary to develop differential stains or modify some of the present protozoan solutions so as to adapt them to plant material. Much more desirable would be the growing of these flagellates in pure culture and staining them in the absence of the host-plant tissues.

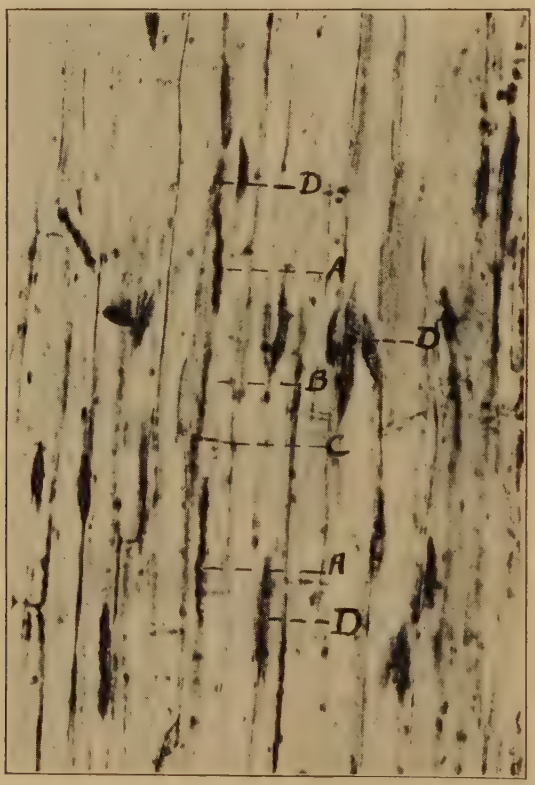

FIG. 15. Longitudinal section through the phloem of a potato plant affected with leaf-roll. Two elongated, slender protozoan organisms are shown in adjoining sieve tubes. × 650.
A. Protozoan organisms.
B. Sieve tube.
C. Sieve plate.
D. Cell nucleus.

Potato Leaf-Roll

The study of material from potato plants affected with leaf-roll has been carried out in the same way as for bean and tomato mosaic. The plants were first carefully examined to exclude if possible any that might also be affected with mosaic. The plants used for this study were taken from a small patch of potatoes where no mosaic was found. They showed unmistakable symptoms of leaf-roll, the lower leaves being uprolled, rigid, brittle, and much thickened, while the entire plant was stunted in growth. Pieces of stem and

FIG. 16. Photomicrograph of a protozoan coiled around a sieve tube nucleus in a potato plant affected with leaf-roll. × 1500.
A. Trypanosome.
B. Nucleus.
C. Sieve tube.
D. Elongated protozoan partially out of focus.

petiole from near the top of the plant were cut and fixed. Some difficulty was experienced in cutting good sections with phloem and xylem tissue intact.

Lying in the sieve tubes of petioles and stems, long, trypanosome-like organisms were found which stained in much the same manner as the bean and tomato organisms. They vary as much as do the types found in tomato mosaic plants, but are, nevertheless, characteristic and constant for this disease. On account of their distribution in the plant and the tendency to distribute themselves along only certain of the sieve tubes they are not always found

readily. They may be present in only one or a few sieve tubes in a bundle, so that in examining longitudinal sections it is necessary to locate the region where they are present. Once they are located they can be found in almost an unbroken succession of cells along the stem or petiole. Two adjoining sieve tubes are shown in Fig. 15 with the organisms in each of them. A great many of these flagellates are found in contact with the nucleus, at least part of the body being in contact with the nuclear membrane. Many times they have been observed actually coiled around it. Such a position of the organism is shown in Fig. 16. This intimate contact with the nucleus is

FIG. 17. Photomicrograph of longitudinal section through potato petiole tissue from leaf-roll plant, showing characteristic trypanosome-like organism that is associated with this disease lying in a sieve tube. × 1500.
A. Sieve plate.
B. Trypanosome.
C. Sieve tube.
D. Cell nucleus.

more pronounced and more common than has been observed with the organisms found in tomato and bean mosaic. Many of them, however, are free within the host cell and are found at various places in the cell. Their position is nearly always parallel with the long axis of the cell, although smaller forms are occasionally seen lying obliquely. A large organism is

shown in Fig. 17, while a shorter, broader and more sinuous form is seen in Fig. 18. Some of the forms observed are very long and narrow, being less than 1 micron in width. These very slender forms can undoubtedly pass through the sieve plate perforations, although none have been seen in this position.

These organisms appear to possess a distinct undulating membrane, and occasionally an individual has been seen with one end of the body drawn out into a rather long flagellum. The membrane is not clearly visible the entire length of the body so that the point of attachment has not yet been observed. Dark staining granules, very distinct, are located at each end of the body. They are probably blepharoplasts and it seems probable that an undu-

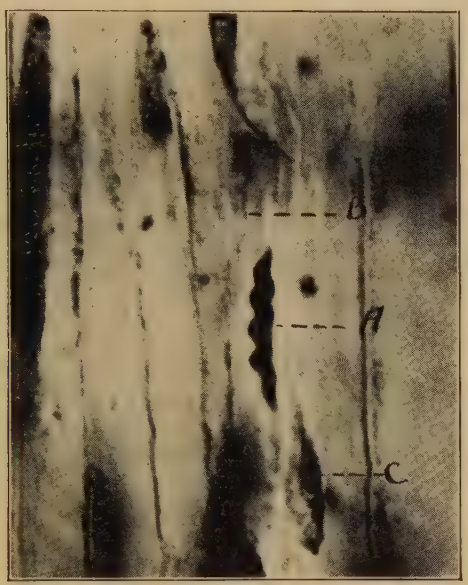

FIG. 18. Photomicrograph of a shorter type of trypanosome found in sieve tubes of leaf-roll potato plants. Note that there appears to be a membrane visible on the right side of the body × 1500.
A. Trypanosome.
B. Sieve tube.
C. Sieve tube nucleus.

lating membrane runs the entire length of the organism. However, since imagination may easily outrun perception in matters of detail, structural characters will require further study. Extreme cases of body sinuosity are common among these organisms and it is in these individuals that an undulating membrane is most often seen. Some details observed in slides studied include: deep-staining granules in various parts of the organisms; the presence of vacuoles; variation in intensity of staining of various parts of the body, in some cases giving a banded appearance to the flagellate. This is seen in Fig. 16. A centrally located nucleus has not been constantly found in the flagellates examined. For a study of nuclear details other stains will have to be used.

The size of these organisms is very variable. The measurements given in the table below show the limits of variation that have been observed:

Longest form measured	35 microns
Shortest form measured	11.3 microns
Broadest form measured	3.0 microns
Slenderest form measured	0.9 micron
Average all measurements	23.6 × 1.8 microns

Smaller individuals than those given in the table above have been seen, but on account of their position in the cell have not been measured. Some are coiled around the nucleus, others occupy positions such that a portion of the body is obscured, so that the measurements given in the table, while representative of a majority of individuals, can not be said to show the extreme variation in size. Further study and measurements of a large number of individuals must be made before any definite conclusions concerning these points can be made.

For a study of healthy tissues sections were made from plants that showed no symptoms of leaf-roll. No organisms have been found in the sieve tubes of these plants in all the slides examined. From all the evidence gathered in this study of diseased and healthy material it seem safe to conclude that these organisms occur in the sieve tubes of diseased plants but are absent from these structures in healthy ones.

Nature of the Organisms

From the study that has been made of the organisms associated with the diseases investigated there seems little doubt of their protozoan nature. Their simple unicellular structure, possession of flagella and undulating membranes, the presence of kinetonuclei and other characteristics that coincide with the peculiarities of the protozoa, seems sufficient proof of the true nature of the forms found in the phloem tissues of plants affected with mosaic and similar diseases. The systematic position of these organisms is a question requiring more detailed study. While not final, the evidence now seems to warrant a few conclusions, which may be considered tentative until further study shall place these forms in their proper generic position. The biflagellates associated with bean and clover mosaic are apparently a new form, since no type of protozoan similar to it has been described. It differs from other probably closely related types, like Leptomonas, in the arrangement of the flagella. The Leptomonas organisms found in the latex of Euphorbiaceous and Asclepiadaceous plants are typical members of that genus with a single polar flagellum, while the bean organism has one flagellum attached to each pole, a characteristic that is constant in all the material examined.

The great resemblance of the organisms found in mosaic tomato plants to trypanosomes is unmistakable. While there is great variation in the morphology of various individuals the general characteristics are more nearly like those of trypanosomes than any other form. The great polymorphism shown by the different species included within the broad limits of the genus Trypanosoma admits forms which might possibly belong in other subdivisions that have been proposed for this genus. They have, however, not been generally accepted by protozoologists. Certain details of structure have not been demonstrated with the stains that have been used, so that for the

present the systematic position of the tomato organism must be left open for further study.

The potato leaf-roll organism is a protozoan which even more strongly resembles members of the genus Trypanosoma. The sinuous form of the body and the occurrence of an undulating membrane are characters which belong only to organisms of this type. That other forms also occur in the life cycle is very probable. Some of the long slender organisms found in the sieve tubes have a great resemblance to spirochaetes. They are, however, much larger than any known species, and slender trypanosomes have often been mistaken for spirochaetes by protozoologists. The recent work of Mesnil with *Leptomonas Davidi*, a parasite in the latex of Euphorbiaceous plants, has shown that these organisms exhibit great polymorphism, some individuals being typical Leptomonads, while others more nearly resemble trypanosomes. It is probable, therefore, that the potato leaf-roll organism will be found to contain quite widely varying forms in its life-cycle.

OTHER KNOWN PROTOZOAN DISEASES OF PLANTS

Lafont (6), Franca (7), Mesnil (8), Franchini (9), and other European workers have found protozoa in plants belonging to Euphorbiaceae, Asclepiadaceae and Urticaceae. Typical Leptomonad forms have been found in the latex of Euphorbiaceous plants, and these organisms are constantly associated with a disease of the plants, characterized by etiolation and wilting. Transmission of the flagellates has been shown to occur through the agency of sucking insects (Coreidae). In the bodies of these insects the protozoan passes a portion of its life-cycle, eventually developing non-flagellate forms which are found in the salivary glands. Flagellate Leptomonad and Trypto-monad forms have been found in the latex of *Ficus parietalis*, as have also some characteristic amoeboid organisms.

The measurements of the flagellates from the latex of the Euphorbiaceous plants very nearly coincide with those found in bean plants affected with mosaic. The mono-flagellate character of the organisms from the latex of these plants indicates that these are distinct from the organisms associated with bean and clover mosaic.

The demonstration of protozoa as the cause of plant disease had its inception in 1910 in the work of Lafont (6) who found the Leptomonad forms in the latex of certain Euphorbiaceous plants. This stands as the first record of a plant disease caused by a protozoan parasite. That a great many other plant diseases of unknown cause, will, upon investigation, yield similar results, is not unlikely from the evidence now available of the relation of these simple organisms to plant disease.

PROBABLE RELATION OF THE ORGANISMS TO THE DISEASES WITH WHICH THEY ARE ASSOCIATED

Since the description of tobacco mosaic by Mayer (15) in 1886, the cause of this and other mosaic diseases has been a question of paramount interest to phytopathologists. There is no longer any doubt of the infectiousness of material from diseased plants, and that the power of such a small amount of material to produce disease when introduced into healthy plants is a characteristic of some "living contagium" seems evident from the work of Allard (14) and others. The fact that insects carry and transmit the causative agents of these diseases is itself significant, because of the known facts of insect transmission of protozoan animal and human diseases. Further, the paucity of definite experimental proof of the absence of visible organisms in plant tissues affected with mosaic, is evident from an examination of the literature. Finally, the apparent ultra-microscopic size of infectious particles which pass through the bacterial filters seems to have been the barrier which has hitherto deterred extensive attempts by cytological methods to demonstrate visible organisms in constant association with mosaic plants.

In this investigation the finding of definite protozoan organisms in constant association with mosaic plants and their absence from healthy ones indicate that they are probably the factor so long sought as the cause of these diseases. The fact that the mosaic diseases are cross-inoculable among groups of closely related plants, and the finding of similar organisms in mosaic bean and clover plants makes this seem even more probable.

The work of Elmer (10), who claims to have obtained successful results in cross-inoculation studies of mosaic from Cucurbitaceae, Solanaceae, and Leguminosae, will need extensive confirmation under carefully controlled conditions, especially since other investigators have reported negative results from similar attempts, and also because of the diversity of form shown by the flagellates from diseased plants in these different families. The possibility of great polymorphism within the same species, and the adaptation of organisms to changed conditions must, however, also be kept in mind. Until these organisms can be isolated and grown in pure culture and inoculated into healthy plants, the evidence of their causal relation to the diseases in question necessarily will be circumstantial. Such studies are now in progress and also cytological work in an attempt to find in the aphids which transmit the causative agents, forms belonging in the life-cycle of these flagellates. Success in this last venture would greatly strengthen the evidence now available.

Concerning the filterable nature of the infectious agents of mosaic diseases, investigation is much needed of the process by which colloidal and other particles pass through the pores of these bacterial filters. Some of the very slender forms of the bean flagellates, less than 0.3 micron in diameter, might pass through the medium filters. There is also the possibility of "symplastic" forms somewhere in the life-cycle. In this connection Schaudinn (11) has expressed the opinion that some stages of trypanosomes investigated by him were small enough to pass through bacterial filters. Moore and Breinl (12) have asserted that infected blood retained its infectiousness after filtration. The work of Fry (13) with *Trypanosoma brucei* is in line with these obser-

vations. He states that *T. brucei* can throw off particles which, when liberated in the blood, have a motility of their own. This extrusion of granules he regards as of a vital and not degenerative nature. The filterable nature of certain spirochaetes which, in size, are comparable to the small flagellates present in bean mosaic, and the possibility, as expressed by some bacteriologists, that the organisms which are said to *pass* through bacterial filters, actually *grow* through the pores, disclose a field for investigation, which undoubtedly will yield important results.

SUMMARY

Using modern cytological methods, protozoan killing and fixing solutions and protozoan stains, an intensive study has been made of bean mosaic, clover mosaic, tomato mosaic and potato leaf-roll. Definite protozoan organisms, located mainly in the sieve tubes and sieve parenchyma, have been demonstrated to be constantly associated with these diseases.

The bean and clover organism is a biflagellate, elongated protozoan whose generic position is near Leptomonas. It apparently is a flagellate of new generic rank, since the location and attachment of the flagella differ from the structure in any known genus.

The organisms found in mosaic tomato plants apparently are trypanosomes, or are closely related to this genus. They have been found only in the sieve tubes. Their size varies from 6 to 30 microns in length and from 0.5 to 6 microns in width.

In the sieve tubes of potato leaf-roll plants, organisms have been found which more closely resemble trypanosomes than any other form. They are usually closely associated with the host cell nucleus. These organisms are also variable in size, some individuals being less than 1 micron wide and 12 long, while the longest one measured was 35 microns.

All of these organisms lie in a plane parallel to the long axis of the cell and have been demonstrated only in longitudinal sections.

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